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STUDIES ON ACID HYDROLASES
III. ISOLATION AND PROPERTIES OF SPLEEN ACID RIBONUCLEASE

ALBERTO BERNARDI AND GIORGIO BERNARDI
Centre de Recherches sur les Macromolécules, Strasbourg (France)

SUMMARY

A procedure for the preparation of spleen acid ribonuclease is described.

The enzyme has a sedimentation constant close to 2.3 S. It is thermolabile and has a pH optimum of 5.3. The hydrolysis of soluble RNA by the enzyme proceeds only slightly more slowly than that of ribosomal RNA. Among synthetic polyribonucleotides, polyuridylic acid is hydrolyzed rapidly, polyinosinic acid slowly; polycytidylic and polyadenylic acids are highly resistant to the enzyme.

INTRODUCTION

Acid ribonuclease activity is very widely distributed in different tissues and species; evidence is available that this enzymatic activity is due to more than one enzyme[1]. The purification of the enzymes responsible for this activity has been attempted in only a very few cases, and very often the comparison of the partially purified enzyme preparations reported so far is difficult. One of the best characterized acid ribonuclease preparations is the partially purified enzyme from calf spleen described by MAVER *et al.*[2,3]; the purification of an acid ribonuclease from human spleen has been also reported[4].

We describe here a procedure for obtaining a chromatographically homogeneous acid ribonuclease from hog spleen. The method used is derived from that already reported for the isolation of acid deoxyribonuclease[5]. Several of the properties of the enzyme preparation, as obtained by this method, have been investigated.

EXPERIMENTAL PROCEDURE

Materials

These have already been described[5–7]. High molecular weight ribosomal RNA from yeast was a preparation from Prof. EBEL's laboratory; it was obtained according to the method of MONIER *et al.*[8] as modified by WEIL *et al.*[9]. Synthetic polyribonucleotides were commercial products (Miles, Clifton, N.J.).

Column chromatography and sucrose-gradient centrifugation

These were carried out as described elsewhere[5–7,10].

Assay of enzymatic activities

The acid ribonuclease activity was assayed by measuring the liberation of acid-soluble oligonucleotides from soluble RNA, which is an excellent substrate for this enzyme. The reaction mixture (total volume 1.25 ml) contained: (a) 0.8 μmoles sRNA-*P* (General Biochemicals, Chagrin Falls, Ohio), 187.5 μmoles acetate buffer (pH 5.0), 12.5 μmoles EDTA. (b) enzyme; if necessary, this was diluted with 0.15 M acetate buffer–0.01 M EDTA (pH 5.0) containing 0.05 % Armour bovine serum albumin.

The assay procedure described for acid deoxyribonuclease[5] was used, except that all volumes were halved. Activity units are defined as for acid deoxyribonuclease[5] except that they were not divided by 2. One activity unit, as defined above, corresponds to the liberation of 0.11 μmoles of ribonucleotide phosphorus.

The enzymatic activity of acid ribonuclease on synthetic polyribonucleotides and RNA "core" was determined as indicated above for the acid ribonuclease assay except for the following details. The absorbance of the polyribonucleotide solutions at 260 mμ was equal to 6.7; that of RNA "core" was 19. Precipitation of acid-insoluble oligonucleotides was carried out with 12% perchloric acid[5,6], except in the case of polyuridylic acid and RNA "core" where a uranyl acetate–perchloric acid solution was used as the precipitating solvent[6,11].

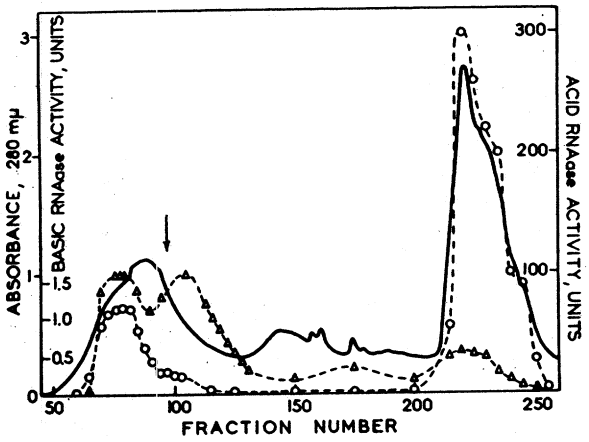


Fig. 1. Chromatography on DEAE–Sephadex A-50 of crude spleen nuclease II. 330 ml of preparation HS 9 ($A_{280m\mu}$ = 10.3; $A_{260m\mu}$ = 6.9) were loaded on a 8 cm × 80 cm column of DEAE–Sephadex A-50 equilibrated with 0.05 M phosphate buffer (pH 6.8). This buffer was also used to elute the first protein peak. 0.5 M phosphate buffer (pH 6.8) was applied at the fraction indicated by the arrow. 24-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand scale). Triangles indicate the basic ribonuclease activity (left-hand inner scale). Acid deoxyribonuclease, cytochrome *c*, acid phosphomonoesterase and phosphodiesterase were also assayed; the results are shown in Fig. 1 of refs. 5 and 7.

"Basic" ribonuclease activity was assayed exactly like the acid activity, except that 0.1 M phosphate buffer (pH 7.2)–0.01 M $MgCl_2$ replaced acetate buffer–EDTA as the solvent[3]. Acid deoxyribonuclease, acid phosphomonoesterase, phosphodiesterase were assayed as described elsewhere[5,7].

RESULTS

Preparation of the enzyme

The starting material was the crude spleen nuclease II preparation[5]. Table I shows the acid and basic ribonuclease activities as determined at the different steps leading to the crude enzyme preparation.

TABLE I

PREPARATION OF SPLEEN ACID RIBONUCLEASE

Values show total acid (columns 1) and basic (columns 2) ribonuclease activities per kg of trimmed spleen as determined on aliquots taken from a preparation at the successive steps indicated in the first column. The supernatants obtained by centrifuging products 1, 2 and 3 at 8000 × *g* for 1 h and the aqueous solution of precipitate 4, were dialyzed against 0.15 M NaCl and assayed. The dry weights of undialyzable material per kg of trimmed spleen, as determined at the successive steps, were reported in Table I of ref. 5.

Preparation step	*Extraction procedure* 0.15 *M NaCl*		0.1 *M HCl*		0.05 *M* H_2SO_4	
	(1)	(2)	(1)	(2)	(1)	(2)
1. Extraction	12 150	960	23 600	4 150	21 300	4 620
2. Acidification (pH 2.5)*	22 600	10 500	26 500	9 050	32 100	15 450
3. 0.4 $(NH_4)_2SO_4$ saturation	—	—	—	—	—	—
4. 0.8 $(NH_4)_2SO_4$ saturation	5 870	880	6 860	800	6 960	740

* This was done with 0.2 M HCl for the extracts obtained with 0.15 M NaCl and 0.1 M HCl.

The acid and basic ribonuclease activities of crude spleen nuclease II were found to be 5000–6000 and 600–700, respectively, per kg of ground spleen. The specific activities were about 20 and 2, respectively. A relatively larger variability of both activities, compared with the other enzymatic activities tested (acid deoxyribonuclease, acid phosphomonoesterase), was noticed when studying different crude spleen nuclease preparations.

The chromatographic purification was largely patterned on Procedure C, already described for acid deoxyribonuclease[5]. The results are summarized in Table II. A description of the chromatographic steps follows.

Step I. DEAE–Sephadex A-50 (Fig. 1). This step has already been described[5]. Two fractions showing ribonuclease activity were not retained by the column equilibrated with 0.05 M phosphate buffer (pH 6.8). The first ribonuclease fraction had a ratio of acid to basic activity close to 40; the second activity peak had a ratio of about 10. These ribonuclease activities are currently being studied in more detail;

TABLE II

CHROMATOGRAPHIC PURIFICATION OF SPLEEN ACID RIBONUCLEASE

The values reported refer to the fractions which were processed further or to the final product.

Fraction	*Volume (ml)*	*Total $A_{260m\mu}$*	*Total units*	*Specific activity*
Crude spleen nuclease II				25*
I. DEAE-Sephadex	70	1820	87 000	48
II. Sephadex G-100	200	680	74 000	109
III. Hydroxyapatite	120	80.5	43 300	538
IV. CM-Sephadex	183	19	41 000	2160
V. Hydroxyapatite	5.1	6.2	15 000	2410

* This is the value obtained for preparation HS 11.

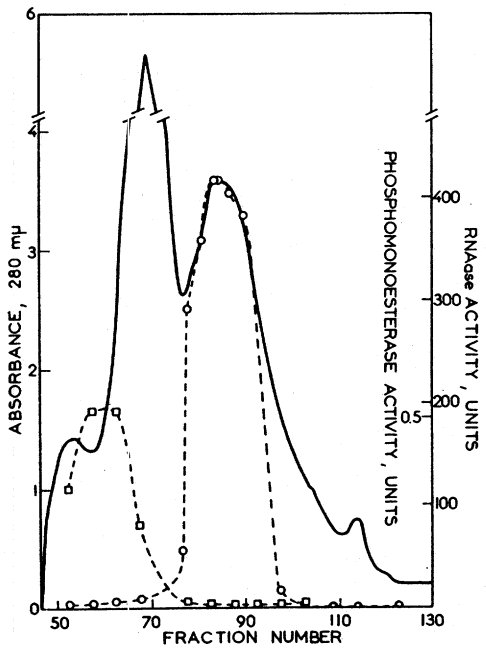


Fig. 2. Gel filtration on Sephadex G-100 of an acid ribonuclease-rich fraction obtained from DEAE-Sephadex. 70 ml ($A_{280m\mu}$ = 26.00; $A_{260m\mu}$ = 15.80) were loaded on a 4 cm × 90 cm column of Sephadex G-100 equilibrated with 0.05 M phosphate buffer (pH 6.8). The same buffer was used for the elution. 10-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand scale). Squares indicate the phosphomonoesterase activity (right-hand inner scale). Fractions 77–96 were processed further.

their chromatographic behaviour on hydroxyapatite is shown in Fig. 2 of ref. 5.

A third ribonuclease activity peak was eluted by 0.5 M phosphate buffer (pH 6.8); the ratio of acid to basic activity in this case was found to be higher than 500. This fraction was processed further and its purification is described here. For the sake of convenience, acid ribonuclease-rich fractions from DEAE-Sephadex

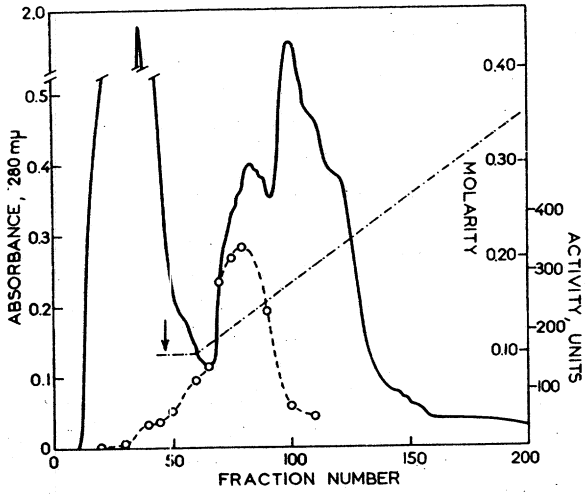


Fig. 3. Chromatography on hydroxyapatite of acid ribonuclease fractions from Sephadex G-100. 200 ml ($A_{280m\mu}$ = 3.40; $A_{260m\mu}$ = 2.78) were loaded on a 2 cm × 40 cm column of hydroxyapatite. Elution was carried out with a molarity gradient (0.1 to 0.5 M) of phosphate buffer (pH 6.8); this was started at the fraction indicated with an arrow (right-hand inner scale). 10-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand scale). No phosphomonoesterase activity was detected in fraction 20–120. Fractions 60–90 were processed further.

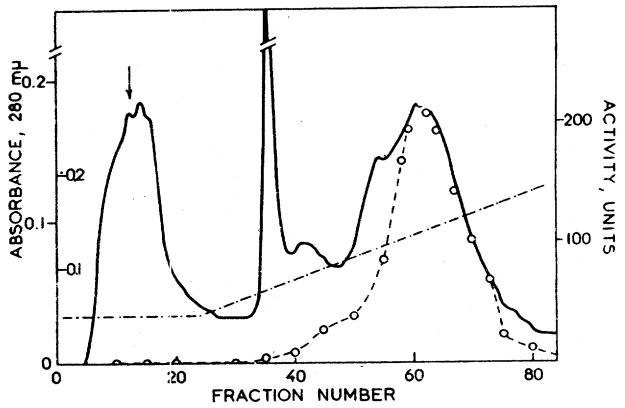


Fig. 4. Chromatography on CM-Sephadex C-50 of acid ribonuclease fractions from hydroxyapatite). 120 ml ($A_{280m\mu}$ = 0.67; $A_{260m\mu}$ = 0.40) were loaded on a 2 cm × 40 cm column of CM–Sephadex C-50 equilibrated with 0.05 M phosphate buffer (pH 6.0). Elution was carried out with a molarity gradient (0.05 to 0.4 M) of phosphate buffer. This was started at the fraction indicated by the arrow (left-hand inner scale). 10-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand scale). Fractions 58–72 were processed further.

columns were precipitated with $(NH_4)_2SO_4$ (0.8 saturation at 20°; solutions were made 0.001 M in EDTA) and stored at 4°. When enough product was available, the precipitate was collected by centrifugation and dissolved in a small volume of distilled water.

Step II. Sephadex G-100 (*Fig. 2*). The acid ribonuclease solution described above was loaded on a Sephadex G-100 column equilibrated with 0.05 M phosphate buffer (pH 6.8). A complete separation of acid phosphomonoesterase and acid ribonuclease activities was obtained by eluting with 0.05 M phosphate buffer (pH 6.8).

Step III. Hydroxyapatite (*Fig. 3*). The active fraction from Sephadex G-100 was loaded on a hydroxyapatite column equilibrated with 0.1 M phosphate buffer (pH 6.8). A molarity gradient (0.1 to 0.5 M) of phosphate buffer (pH 6.8) eluted the ribonuclease activity at a molarity of about 0.12. The active fractions were concentrated to a small volume by freeze-drying and loaded on a Sephadex G-25 column equilibrated with 0.05 M phosphate buffer (pH 6.0).

Step IV. CM–Sephadex C-50 (*Fig. 4*). The ribonuclease fraction from Sephadex G-25 was loaded on a CM–Sephadex C-50 column equilibrated with 0.05 M phosphate buffer (pH 6.0). Elution was carried out with a 0.05 to 0.4 M phosphate buffer gradient (pH 6.0). Acid ribonuclease was eluted after an inactive peak at a molarity of about 0.15. The central fractions from the activity peak were pooled and run on a Sephadex G-25 column equilibrated with 0.05 M phosphate buffer.

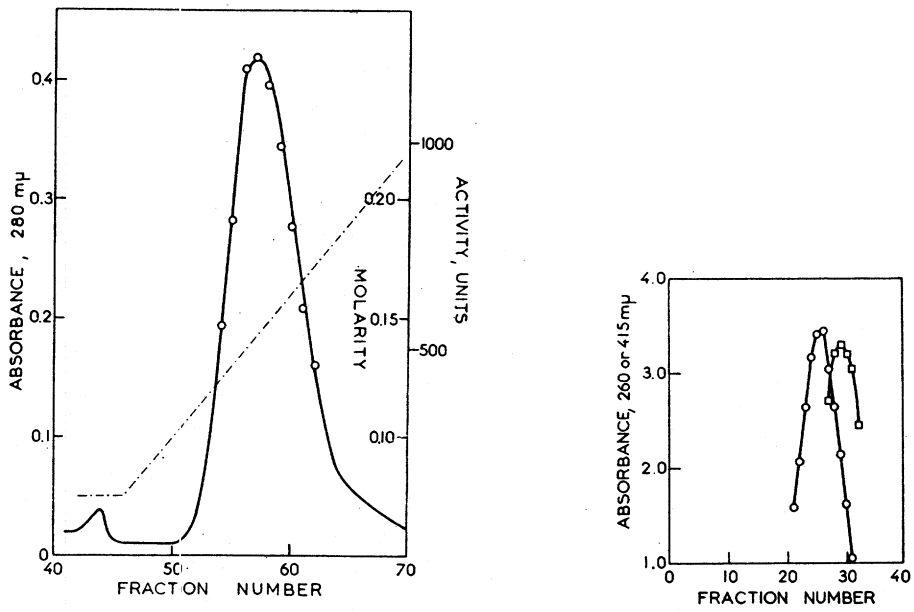
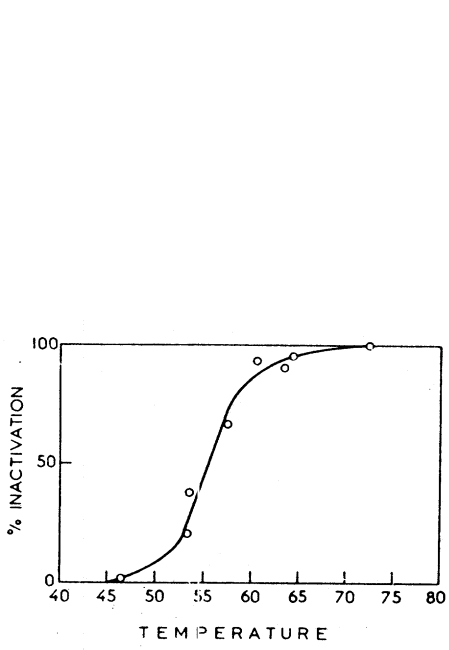


Fig. 5. Chromatography on hydroxyapatite of acid ribonuclease fractions from CM–Sephadex 183 ml ($A_{280m\mu}$ = 0.104; $A_{260m\mu}$ = 0.064) are loaded on a 1 cm × 13 cm hydroxyapatite column equilibrated with 0.05 M phosphate buffer (pH 6.8). A molarity gradient (0.1 to 0.3 M) of phosphate buffer was used to elute acid ribonuclease (right-hand inner scale). 4.6.-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid ribonuclease activity (right-hand scale). Fractions 53–60 were processed further.

Fig. 6. Sucrose-gradient centrifugation of spleen acid ribonuclease; cytochrome *c* was used as a reference protein. A total of 37 fractions was collected. Circles indicate ribonuclease activity; points, the absorbance of cytochrome *c* at 415 mμ. The bottom of the cell is at the left. A linear molarity gradient was obtained by using 5 % and 20 % sucrose solutions in 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA. Centrifugation was carried out for 16 h at 4° at 38 000 rev./min using a SW-39 rotor and a Spinco Model-L ultracentrifuge.



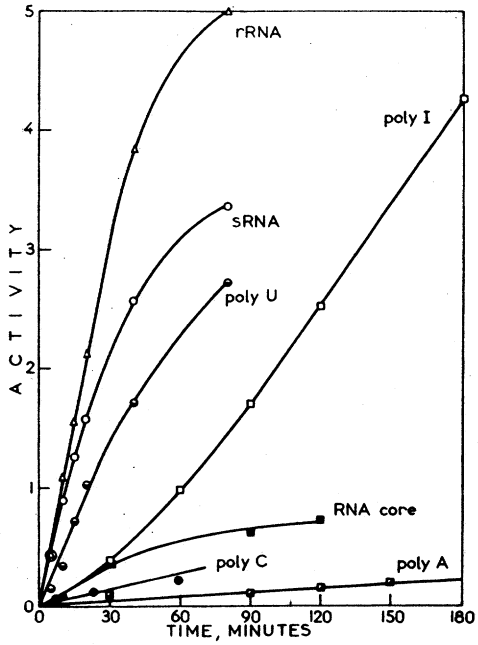


Fig. 7. Thermal inactivation curve. Acid ribonuclease samples in 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA were kept 20 min at the temperatures indicated on the abscissa; they were then chilled in an ice-bath and assayed at 37°.

Fig. 8. Time course of the degradation of polyribonucleotides at 37° by acid ribonuclease. The solvent was 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA. The absorbances at 260 mμ of the polyribonucleotide solutions were equal to 6.7; RNA "core" had an $A_{260m\mu}$ of 19.

Step V. Hydroxyapatite (*Fig.* 5). The ribonuclease fractions from the previous step were loaded on a hydroxyapatite column equilibrated with 0.05 M phosphate buffer (pH 6.8). The ribonuclease activity was eluted by a molarity gradient (0.05 to 0.3 M) of phosphate buffer (pH 6.8) at a molarity of 0.12. The central fractions of the peak showed a constant specific activity. They were run through a Sephadex G-25 column equilibrated with 0.004 M acetate buffer (pH 5.0). The enzyme solution was then concentrated by freeze-drying to an $A_{280m\mu}$ equal to 1.2. The concentrated enzyme solution, referred to as the final product in Table II was then frozen and stored at −60°. Alternatively, the enzyme may be freeze-dried and stored at −60° without any loss in activity.

Properties of the enzyme

Physical properties. The sedimentation constant was determined by centrifuging acid ribonuclease in a sucrose-density gradient according to MARTIN AND AMES[12] cytochrome *c* being used as a reference protein. The results are shown in Fig. 6; a sedimentation constant equal to 2.3 S was calculated. The thermal inactivation curve of the enzyme in 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA is shown in Fig. 7.

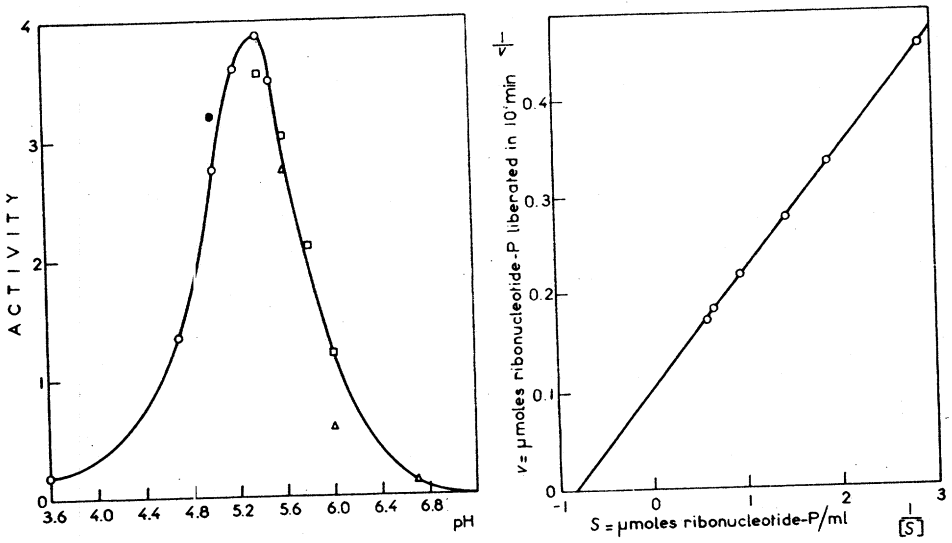


Fig. 9. Acid ribonuclease activity on soluble RNA; pH–activity curve at 37°. The solvents were: 0.10 M NaCl–0.05 M acetate (circles), 0.05 M succinate (squares), 0.05 M phosphate (triangles), respectively. The activity measured when using 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA is given for comparison (point).

Fig. 10. Lineweaver–Burk plot for acid ribonuclease. Soluble RNA was used as the substrate and 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA as the solvent.

Purity. Spleen acid ribonuclease, as obtained by the present procedure, is completely free of the following enzymatic activities: acid phosphomonoesterase, phosphodiesterase, basic ribonuclease, acid deoxyribonuclease.

Enzymological properties. The enzyme hydrolyzes yeast soluble RNA only slightly more slowly than yeast ribosomal RNA (Fig. 8). The final digests of soluble and ribosomal RNA's may be further degraded by using splenic exonuclease[13]. Among the synthetic polyribonucleotides poly-U is attacked at a fairly high rate, whereas poly-C and especially poly-A are hydrolyzed very slowly (Fig. 8). Poly-I is attacked at an intermediate rate as compared with poly-U and poly-C, but the time course of its degradation shows an upward curvature at the early stage of digestion (Fig. 8). RNA "core", the water-undialyzable RNA oligonucleotide fraction resistant to pancreatic ribonuclease, is hydrolyzed by the enzyme. It is difficult, however, to compare the hydrolysis rate for this substrate with those for the other polyribonucleotides since, when used at the same concentration as other substrates, RNA "core" is very soon exhausted.

The pH–activity curve, using soluble RNA as substrate, is shown in Fig. 9. The pH optimum is close to 5.3. In the pH range 7–8.6 the enzyme is completely inactive.

A Lineweaver–Burk plot using soluble RNA as substrate and 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA is shown in Fig. 10. A Michaelis constant $K_m = 1.21 \cdot 10^{-3}$ was calculated from the data.

DISCUSSION

The results presented in Table I clearly show that both acid and basic ribonuclease activities are extracted to a larger extent by acidic than by neutral solutions. This is, however, an overall result which applies to several different enzymatic proteins. A large decrease in both activities, particularly marked for the basic activity, occurs when proceeding from the acidification step to the final $(NH_4)_2SO_4$ precipitation. It is very likely that this phenomenon is due to a fractionation of different ribonucleases.

The sedimentation coefficient found indicates that spleen acid ribonuclease is a small protein with a molecular weight probably lower than 20 000. The enzyme molecule is quite resistant to surface denaturation and may be freeze-dried without loss of activity; the enzyme is, however, thermolabile and 50 % of its activity is destroyed by a 20-min treatment at 55°.

The results on the digestion of natural and synthetic polyribonucleotides reveal several interesting features. The first is the only slight difference in the hydrolysis rates found for soluble and ribosomal RNA; this behaviour is quite different from that shown by other ribonucleases, particularly by pancreatic ribonuclease. The very different hydrolysis rates found for the synthetic polyribonucleotides seem to be related to their secondary structure, since polyuridylic acid, which is devoid of any secondary structure, is attacked at a much faster rate than polycytidylic and polyadenylic acids which have a helical structure under the experimental conditions used. This point, as well as the abnormal behaviour of poly-I (Fig. 8), are now being investigated further. The properties manifested so far by our spleen acid ribonuclease suggest that it may be identified with the enzyme present in the partially purified preparation of MAVER AND GRECO[3]. An investigation on the terminal digest of spleen acid ribonuclease is in progress with the aim of elucidating the specificity of this enzyme.

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